Inflammation, Vol. 39, No. 6, December 2016 (© 2016)
DOI: 10.1007/s10753-016-0431-2

ORIGINAL ARTICLE

Contributory Anti-Inflammatory Effects of Mesenchymal Stem Cells, Not Conditioned Media, On Ovalbumin-Induced Asthmatic Changes in Male Rats

Mahdi Ahmadi,[1] Reza Rahbarghazi,[2] Sina Soltani,[2] Mohammad Reza Aslani,[1] and Rana Keyhanmanesh[3,4]

ABSTRACT—Our aim in selecting an appropriate cell fraction and conditioned media (CM) was to achieve the suitable candidate for ameliorating long-term chronic asthmatic changes of respiratory tract. Thirty-six rats were classified into healthy and sensitized groups, which were further divided into three subgroups; rats received systemically 50 μl volume of PBS, CM, or 2×10^6 rat bone marrow-derived mesenchymal stem cells (rBMMSCs). Tracheal responsiveness (TR), immunologic responses, and recruitment of rBMMSCs into the lungs were evaluated. A high degree of TR and total WBC and percentages of eosinophils and neutrophils was significantly recorded in all sensitized groups rather than of controls ($p < 0.001$ to $p < 0.05$). Concurrently, a significant improvement of TR and eosinophil and neutrophil return toward normal levels was evident in sensitized rats receiving cells as compared to parallel asthmatic animals. Flow cytometric monitoring of lymphocyte subpopulation revealed a decrease in the number of $CD3^+CD4^+$ and concurrent increase in $CD3^+CD8^+$ in all sensitized rats as compared to control ($p < 0.001$ to $p < 0.05$). Noticeably, no significant modulatory effects of either cell or CM administration were achieved on the $CD3^+CD4^+$ and $CD3^+CD8^+$ populations in non-asthmatic rats. Corroborating our results, the number of $CD3^+CD4^+$ tended to increase ($p < 0.05$) which coincided with a decreased manner of $CD3^+CD8^+$ populations as compared to other asthmatic groups ($p < 0.01$ to $p < 0.05$). Moreover, stem cells could efficiently transmigrate to the lung parenchyma, albeit the dynamic of asthmatic changes stimulated the rate of recruited cells. Our study shed light on superior effects of mesenchymal stem cells, but not CM, in attenuating chronic asthmatic changes in the model of rat.

KEY WORDS: asthma; inflammation; conditioned media; mesenchymal stem cells; lymphocyte.

INTRODUCTION

Asthma is conceived as a chronic lung disorder with a very high socioeconomic burden, characterized by reversible obstruction, hyper-responsiveness, aberrant remodeling, and noticeable airway inflammation [1, 2]. Based on previous experiments, numerous inflammatory cells from different lineages such as eosinophils, T cells, neutrophils, macrophages, and mast cells could infiltrate to inflamed niche and initiate a sequential stepwise of airway inflammation exerted via secretion of mediators [3, 4]. Asthma *per se* is not curable by any current drugs and convenient strategies, including anti-inflammatory agents and potent bronchodilators, which are used to suppress or alleviate asthma-related symptoms, albeit some relevant side effects impose to affected individuals [5–7]. Since the prominent and problematic characteristic of asthma is

[1] Department of Physiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[2] Stem Cell Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[3] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[4] To whom correspondence should be addressed at Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. E-mail: rkeyhanmanesh@gmail.com

well established by the inflammation of airway, the main solution for treatment of this disorder is focused to control the severity and period of the aforementioned process [7, 8].

Of note, new emerging policies have been undertaken not only to sedate asthma-related complications but also to trigger the regeneration of chronic changes [9–11]. In line with this thought, different stem cells (SCs), particularly mesenchymal stem cells (MSCs), are touted as a population of multi-potent cells, which are found in all adult tissues constituting approximately less than 0.01 % of total cells [12]. In addition to generally defined inherent plastic adherent, self-renewal properties and fibroblast-like appearance, they are able to differentiate into a variety of mesenchymal lineages under appropriate controlled conditions [13]. Until the present time, there is controversy against the role of MSCs on the inflammation procedure. For instance, Abreu *et al*. previously showed a remarkable reduction in immune response coincided by the decrement of collagen fiber content and eosinophil infiltration rate in the chronic murine model of asthma [10]. The cell administration immediately after sensitization resulted in appropriate immune system responses with a shift in T helper 2 to 1 phenotype [14]. In contrast, Trzil and colleagues already affirmed the lack of attenuating properties of adipose-derived MSCs in a feline model of chronic allergic asthma [15].

Two main contributory effects of MSCs, including paracrine activity and trans-differentiation capacity, have been previously documented in target tissues [16]. With regard to limiting factors related with SC administration, the lack of total costs, immunologic reactions, tumorigenicity, and waiting time for cellular expansion makes paracrine soluble factors more suitable for regeneration of target tissue [17].

As far as we know, there is no experiment to assess both juxtacrine and paracrine therapeutic effects of bone marrow-derived MSCs monoculture and MSC-derived conditioned media (CM) separately on immunologic response and $CD4^+$ and $CD8^+$ cells as a rat model of asthma. Our aim in selecting an appropriate cell fraction and CM was to achieve the suitable candidate for ameliorating long-term chronic asthmatic changes of respiratory tract and to decipher fundamental mechanisms of the inflammation in allergic asthma pathophysiology.

MATERIALS AND METHODS

Animal Ethics

All procedure phases of the current experiment were identical to the published guideline of The Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and approved by the Animal Care Committee of Tabriz University of Medical Sciences (No. TBZMED.REC.1394.386).

Cell Isolation and Expansion Procedure

Rat bone marrow-derived mesenchymal stem cells (rBMMSCs) were isolated and expanded as previously described by different authorities [18]. In brief, rats were humanely euthanized by an overdose combination of xylazine and ketamine followed by complete removal of femurs. Afterwards, the epiphysis part of each femur was cut and medullary components immediately flushed out by PBS solution containing 2 % fetal bovine serum (FBS; Gibco, USA). Next, bone marrow mononuclear cells were isolated and enriched by Ficoll (Sigma, USA) gradient centrifugation at 400×*g* for 20 min and then washed twice by PBS solution. The cellular pellet was resuspended in Dulbecco's modified Eagle's medium low glucose (DMEM/LG; Gibco, USA) containing 20 % FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin (Biosera, UK). Ultimately, an initial seeding density of 1×10^5 cells was plated in each square centimeter of six-well plates (SPL). The exhausted medium was further replenished every 4 days and passaged after reaching 70–80 % confluency. The cells at third passage were subjected to current experiment.

Immunophenotyping Characterization of Isolated rBMMSCs

To further confirm the stemness characters of isolated cells, rBMMSCs after third passage were immunophenotypically assessed by BD FACSCalibur flow cytometer (USA) [19]. A panel of antibodies directed against stem-related markers, including FITC-conjugated anti-CD133 (eBioscience, USA), CD44 (eBioscience, USA), and CD34 (eBioscience, USA) as well as PE-conjugated anti-CD31 (eBioscience, USA), was provided. In addition, relevant

isotopes control antibodies were also used to normalize non-specific background and binding. Briefly, the cells were trypsinized by 0.025 % Trypsin-EDTA solution (Gibco, USA), washed twice by PBS, and blocked by 1 % BSA (Sigma, USA) for 15 min. Thereafter, recommended antibody concentration for each abovementioned marker was added into 100 μl of PBS containing 5×10^5 cells, incubated for 30 min at RT, and then washed by PBS. Ultimately, cells were subjected to flow cytometry system, and final output data were analyzed by Flow Jo software ver.7.6.1.

rBMMSCs Labeling with CM-Dil

To monitor the recruitment and homing of rBMMSCs into lungs of sensitized rats, the cells were trypsinized and labeled with 2 μM Cell Tracker™ CM-Dil for 20 min at 37 °C (Catalog No. C-7000; Molecular Probes, Invitrogen, USA). Thereafter, a final volume of 50 μl PBS containing 2×10^6 CM-Dil pre-labeled cells per rat was gently administrated intravenously.

Conditioned Media (CM) Harvesting

To address the possible role of rBMMSCs in the alleviation of ovalbumin-induced asthma in rats via a paracrine manner, the CM produced by rBMMSCs was prepared. In short, after 70–80 % confluency, the DMEM/LG supplemented with 10 % FBS was discarded, washed three times with PBS, and incubated with DMEM/LG-free FBS for 72 h [20]. Next, the supernatant was collected, centrifuged at 400×*g* for 5 min, filtered by 0.20-μm-pore syringe filter, and further concentrated approximately 50-fold by centrifuge filter tubes with molecular weight cut-offs at 4 kDa (catalog no. 003099.125; Eppendorf, Germany). Finally, 50 μl per case of concentrated CM was injected intravenously [10].

Animal Sensitization and Animal Groups

Forty mature male Wistar rats, weighing 200–250 g, were enrolled to the current experiment. The animals were kept in PVC cages under 12:12 light/dark cycle at 20 ± 2 °C with free access to standard rat chow and water. After acclimatization, four rats were randomly selected for extraction and characterization of rBMMSCs prior to the sensitization procedure. The remaining 36 rats were randomly subdivided into six groups ($n = 6$ for each group) as follows:

1. Healthy rats only received 50 μl PBS (C)
2. Healthy rats received 50 μl CM (CSV)
3. Healthy rats 50 μl PBS containing 2×10^6 rBMMSCs (CCV)
4. Sensitized rats only received 50 μl PBS (S)
5. Sensitized rats received 50 μl CM (SSV)
6. Sensitized rats received 50 μl PBS containing 2×10^6 rBMMSCs (SCV)

In the sensitized groups, rats were challenged with ovalbumin (OA) over a period of 32 ± 1 days according to the method described previously [21]. Briefly, rats were injected with 1 mg ovalbumin (Sigma-Aldrich, USA) and 200 mg aluminum hydroxide, dissolved in 1 ml saline, intraperitoneally on the first and eighth days. From the 14th day, the sensitized rats were exposed to an aerosol of 4 % ovalbumin, created by nebulizer (CX3; Omron Co., Netherlands), for 18 ± 1 consecutive days, for 5 min per day. This exposure was performed in a closed box with dimensions $30 \times 20 \times 20$ cm^3 (Fig. 1). In healthy parallel rats, saline was administered instead of ovalbumin in the same manner [2]. One day after sensitization, the sensitized rats were subjected to the experimental procedure of cell or CM administration that was separately injected through the left femoral vein. Calling attention, many authorities acclaimed that the respiratory responses directed to ovalbumin-induced sensitization last over a course of 2 weeks [22, 23].

Tissue Sample Preparation

Two weeks after administration, all animals were anesthetized by the combination of ketamine (75 mg/kg) and xylazine (3 mg/kg) intraperitoneally [24]. Shortly, after opening the chest, their tracheas were gently removed and subsequently sliced longitudinally into two equal pieces of 5 mm. Thereafter, each slice was horizontally mounted between two stainless steel hooks. The lower hook was anchored at the base of the organ bath (Schuler organ bath type 809, Germany) and the next one attached to an isometric transducer (MLT02020, AD Instruments, Spain) [25]. The organ bath was fully filled with 20 ml of Krebs solution and oxygenated consistently with 95 % O_2 and 5 % CO_2 gas mixture at 37 °C. To establish a steady spontaneous tone level, prior to drug additions, a passive tension of 1 g was also applied for at least 60 min.

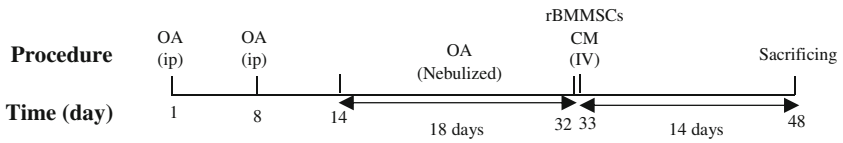

Fig. 1. Flowchart and timeline of the study design. *OA* ovalbumin, *ip* intraperitoneally.

Throughout this period, the exhausted Krebs solution was replenished every 15 min. The changes in isometric force were measured by a transducer coupled to a Power Lab (ML-750, 4 channel recorder; March-Hugstetten, Germany), amplified by an amplifier, and final raw data was interpreted [26].

The Assessment of Tracheal Responsiveness to Methacholine

In each measurement, a dose-dependent response curve of methacholine-induced contraction of a tracheal piece was constructed. In short, cumulative concentrations (10^{-7} to 10^{-3} mM) of methacholine were added consecutively to the bath chamber every 3 min, and the response elicited by each additional dose was recorded after the contraction of the tracheal smooth muscle had plateaued. To construct the curve, the percentage of contraction caused by each methacholine concentration in proportion to the maximum contraction obtained by its final concentration was plotted against log concentration of methacholine. Thereafter, EC_{50} as the effective concentration of drug generating 50 % of the maximal response was expressed in each experiment using methacholine response curves. The contractility response to 10 μM methacholine was also measured as the magnitude of tracheal contraction [27].

Lung Lavage Fluid (LLF) and its Total and Differential Leukocyte Count

After removing the trachea, the lavage was gently performed using 1 ml of normal saline with ten times repetition via a catheter which was inserted into the remaining part of the trachea. A volume of 0.5 ml of collected fluid was stained with the same volume of Turk solution (1:1 dilution), and total leukocytes were counted microscopically in a Neubauer chamber. Turk solution consisted of 1 ml of glacial acetic acid, 1 ml of Gentian violet solution 1 %, and 100 ml distilled water.

The rest of the lung fluid was centrifuged at 2500×*g* for 10 min at 4 °C. The supernatant was discarded, and the thin smear of cells was prepared and stained with Wright-Giemsa stain. Finally, according to cellular morphological characteristics, the percentage of differential leukocytes was determined using a light microscope [28].

Total and Differential White Blood Cell Count

Prior to tracheal responsiveness or bronchoalveolar lavage, 5 ml blood sample was immediately withdrawn via the abdominal aorta. The blood samples were diluted 1 in 20 with Thoma's solution and counted in duplicate in a Neubauer chamber. To clearly differentiate the cell type, smears on glass slides were stained by Wright-Giemsa solution, then the percentage of each type of leukocyte was calculated under the light microscope [8].

$CD3^+CD4^+$ and $CD3^+CD8^+$ Populations in the Circulatory System

To clearly understand whether the administration of marrow-derived MSCs or intravenous injection of CM could alter the percentage of CD4 or CD8 cells, the blood samples from each group were collected and submitted to flow cytometry assay. In brief, an equal diluted blood with PBS (1:1) was overlaid on the same volume of Ficoll (Sigma, USA) and centrifuged at 400×*g* for 30 min at 4 °C. After washing the harvested mononuclear cell three times with PBS, a panel of antibodies against T lymphocyte sub-population, including FITC-conjugated mouse anti-rat CD3 (eBioscience, USA), PE-conjugated mouse anti-rat CD4 (eBioscience, USA), and CD8 (eBioscience, USA), was used. Cells were incubated with recommended concentration of antibodies for 30 min. Finally, cells were subjected to flow cytometry (BD FACSCalibur, USA) and final output data were analyzed by Flow Jo software ver.7.6.1.

Immunofluorescence Assay

To confirm the presence of injected cells, the isolated pulmonary tissues were embedded in tissue freezing media at the end stage of the experimental procedure, snap-frozen, and sectioned at 5 μm using Cryostat apparatus [19]. After thawing the sections at RT, slides were washed twice (5 min each) and stained with 4′, 6-diamidino-2-

phenylindol (DAPI, 1 μg/ml, catalog # D9542; Sigma-Aldrich, USA) for nuclear counterstaining.

Statistical Analysis

All results were presented as mean ± SEM. The data of three sensitized groups were compared with C group using one-way analysis of variance (ANOVA) with Tukey-Kramer *post hoc* test. Also, the data of the SCV group were compared with the SSV group using the unpaired *t* test. We considered statistical significance at $p < 0.05$.

RESULTS

Immunophenotypical assessment of rBMMSCs after the third passage confirmed the stemness characters of isolated cells. Moreover, histograms showed cells positively stained with CD133 and CD44 (Fig. 2).

Tracheal Response to Methacholine

The concentration-response curve to methacholine was shifted leftward in all of the sensitized groups in comparison with the control group (C), whereas the curve of the SCV group showed a rightward shift in comparison with the cell- and CM-free sensitized animals (S). The curve of the SSV group was more similar to group S, and the curves of the CCV and CSV groups were more similar to group C (Fig. 3).

The mean value of EC_{50} in the tracheal pieces of S (0.75 ± 0.01 μM), SSV (0.78 ± 0.01 μM), and SCV (0.82 ± 0.008 μM) groups were significantly lower than group C (3.6 ± 0.07 μM, $p < 0.001$); however, the mean value of EC_{50} in a tracheal piece of the SCV group showed significant improvement in comparison with group S ($p < 0.01$, Fig. 4). Mean value of EC_{50} in the tracheal piece of the SSV group was significantly lower than of the SCV group ($p < 0.05$).

The contractility response of a tracheal piece to methacholine in all of the sensitized groups was significantly higher than group C ($p < 0.001$ for S and SSV groups and $p < 0.05$ for SCV). The contractility response in the SCV group was significantly lower than group S ($p < 0.01$). Moreover, the contractility response of group SSV was significantly greater than that of the SCV group ($p < 0.01$, Fig. 5).

Total White Blood Cell (WBC) Count

The mean value of total WBC in groups S (11,916 ± 166.17), SSV (11,833 ± 125.61), and SCV (11,016 ±

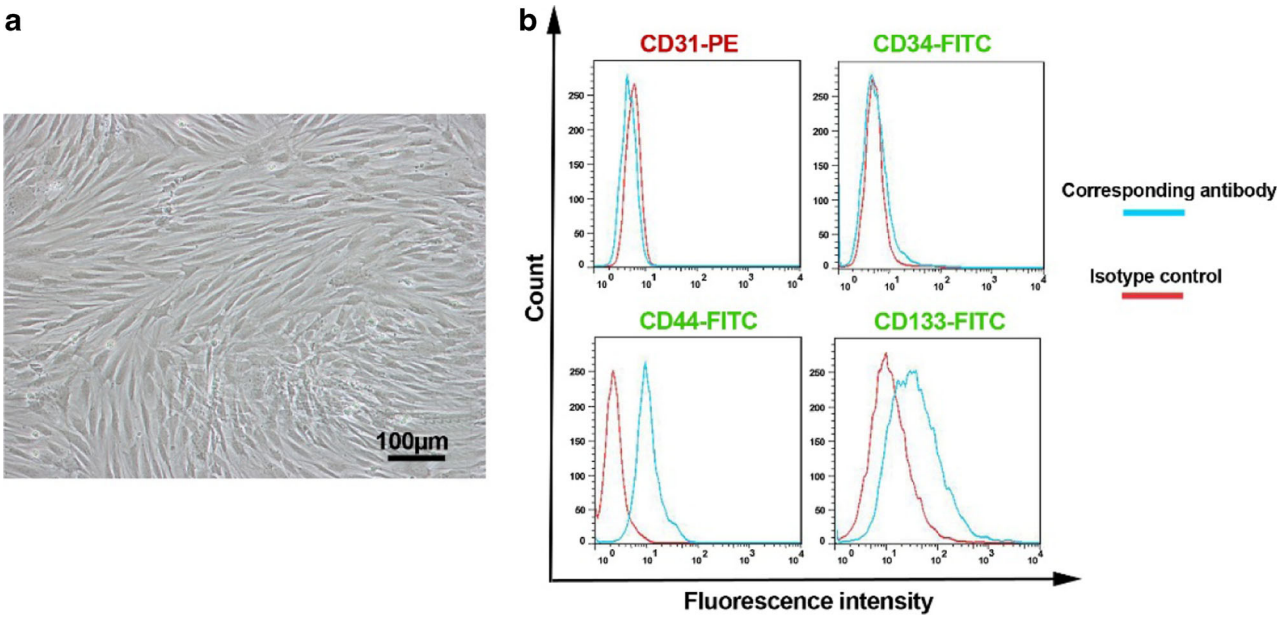

Fig. 2. Phase contrast micrographs of the confluent monolayer of rBMMSCs (passage 3) (**a**). Cultured rBMMSCs' phenotypic characterization (**b**). The expression of both positive and negative markers related to BMMSCs was assessed by flow cytometry analysis. Histograms show cells positively stained with CD133 and CD44.

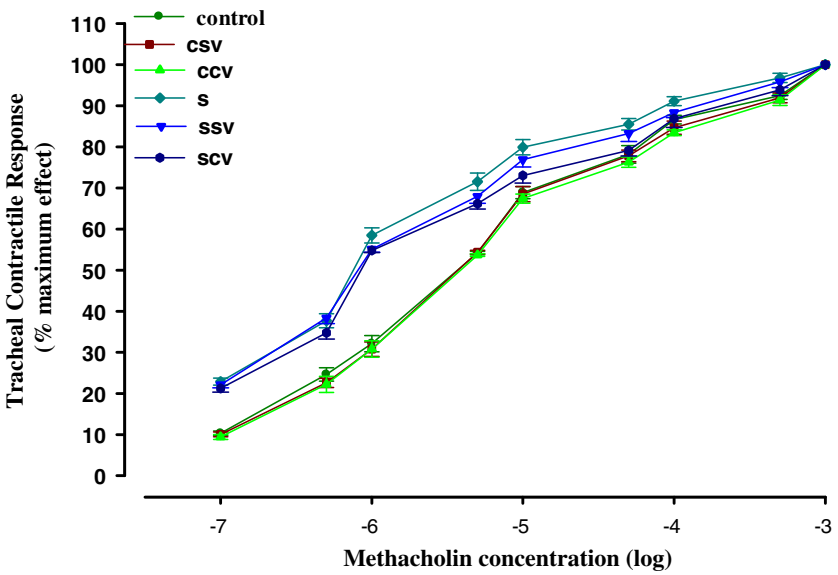

Fig. 3. Cumulative log concentration-response curves of methacholine-induced contraction of isolated trachea in C (control group), CSV (healthy animals received CM), CCV (healthy animals received rBMMSCs), S (sensitized animals), SSV (sensitized animals received CM), and SCV (sensitized animals received rBMMSCs) (for each group, $n = 6$).

153.66) were increased significantly in comparison with group C (5116 ± 94.57, $p < 0.001$ for all). The mean value of total WBC in the SCV group decreased significantly compared to S and SSV groups ($p < 0.01$, Table 1).

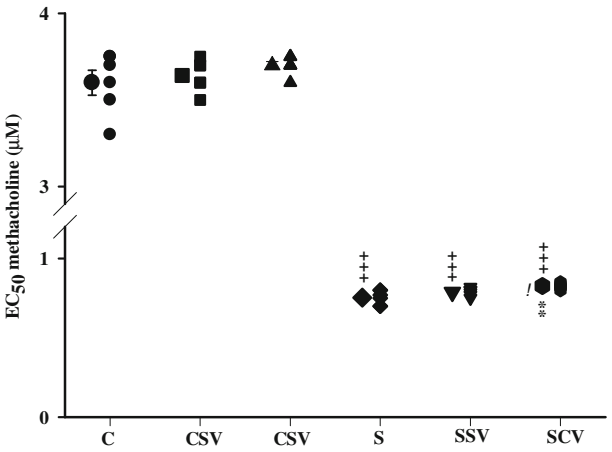

Fig. 4. Individual values and means (*big symbols* with *bars*) of tracheal response to methacholine in C (control group), CSV (healthy animals received CM), CCV (healthy animals received rBMMSCs), S (sensitized animals), SSV (sensitized animals received CM), and SCV (sensitized animals received rBMMSCs) (for each group, $n = 6$). Statistical differences between control and different groups: +, $p < 0.05$; ++, $p < 0.01$; +++, $p <$ 0.001. Statistical differences between SSV and SCV *versus* S group: **, $p < 0.01$; ***, $p < 0.001$. Statistical differences between SSV and SCV groups: !, $p < 0.05$.

Differential Percentages of White Blood Cell (WBC) Count

The percentage of eosinophil in all sensitized groups was significantly higher than group C ($p < 0.001$ to $p < 0.01$). There was a significant decrease in eosinophil

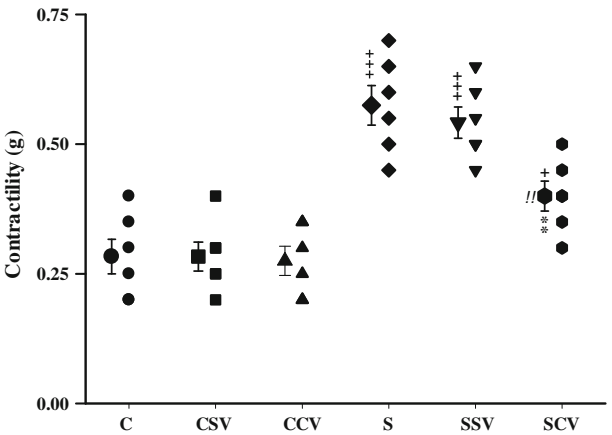

Fig. 5. Tracheal contractility response to 10 μM methacholine in C (control group), CSV (healthy animals received CM), CCV (healthy animals received rBMMSCs), S (sensitized animals), SSV (sensitized animals received CM), and SCV (sensitized animals received rBMMSCs) (for each group, $n = 6$). Statistical differences between control and different groups: +, $p < 0.05$; +++, $p < 0.001$. Statistical differences between SSV and SCV *versus* S group: **, $p < 0.01$. Statistical differences between SSV and SCV groups: !!, $p < 0.01$.

Table 1. Total and Differential Percentage of White Blood Cell (WBC) Count in C (Control Group), CSV (Healthy Animals Received CM), CCV (Healthy Animals Received rBMMSCs), S (Sensitized Animals), SSV (Sensitized Animals Received CM), and SCV (Sensitized Animals Received rBMMSCs) (for Each Group, $n = 6$)

	C	CSV	CCV	S	SSV	SCV
Total WBC (number/mm^3)	5116.67 ± 94.57	5025 ± 118.15	4841.67 ± 133.18	11,916.67 ± 166.17 +++	11,833.33 ± 125.61 +++	11,016.67 ± 153.66 +++ ** !!
Eosinophil (%)	1.1 ± 0.24	1.3 ± 0.23	1 ± 0.4	4 ± 0.56 +++	4.33 ± 0.61 +++	2.6 ± 0.24 ++ * !
Neutrophil (%)	26 ± 0.84	25..66 ± 0.93	24 ± 1.24	31 ± 1.63 +	32 ± 2.44 +	29.66 ± 2.95
Lymphocyte (%)	68.66 ± 0.93	69.66 ± 0.84	70.66 ± 1.02	61 ± 1.4 ++	63 ± 1.47 ++	65 ± 1.09 + *
Monocyte (%)	4.3 ± 0.46	3.6 ± 0.23	4 ± 0.48	2 ± 0.4 ++	1.7 ± 0.4 ++	2.3 ± 0.75 +

Statistical differences between control and different groups: +, $p < 0.05$; ++, $p < 0.01$; +++, $p < 0.001$. Statistical differences between SSV and SCV *versus* S group: *, $p < 0.05$; **, $p < 0.01$. Statistical differences between SSV and SCV groups: !, $p < 0.05$; !!, $p < 0.01$

percentage of the SCV group in comparison with the S and SSV groups ($p < 0.05$).

The percentage of neutrophil in the S and SSV groups was significantly higher than group C ($p < 0.05$). There was a non-significant decrease in neutrophil percentage of the SCV group in comparison with the S group, although it was non-significantly more than in the control.

The lymphocyte percentage in all sensitized groups was significantly lower than group C ($p < 0.01$ to $p < 0.05$). However, there was a significant increase in lymphocyte count of SCV group in comparison with the S group ($p < 0.05$).

The percentage of monocyte in all sensitized groups was significantly lower than group C ($p < 0.01$ to $p < 0.05$). There was a non-significant increase in monocyte percentage of the SCV group in comparison with the S group, but in the SSV group, it was non-significantly lower than the S group (Table 1).

Total Leukocyte Count in Lung Lavage Fluid (LLF)

The mean value of total leukocyte in LLF in groups S (100 ± 2.58), SSV (97.5 ± 2.50), and SCV (90.84.14 ± 1.53) were significantly higher than that of group C (32.5 ± 2.14, $p < 0.001$ for all). The mean value of total leukocyte in the SCV group decreased significantly compared to group S and SSV ($p < 0.05$, Table 2).

Differential Leukocyte Percentage in Lung Lavage Fluid (LLF)

The percentages of eosinophil and neutrophil in all sensitized groups were significantly higher than group C ($p < 0.001$ to $p < 0.05$). There was a significant decrease in eosinophil and neutrophil percentages of the SCV group in comparison with the S and SSV groups ($p < 0.05$). However, the neutrophil percentage in the SSV group was non-significantly increased compared to the S group.

The percentage lymphocyte in all sensitized groups was significantly lower than group C ($p < 0.01$ to $p < 0.05$). There was a significant increase in lymphocyte percentage of SCV group in comparison with S and SSV groups ($p < 0.05$).

The percentage of monocyte in S and SSV groups was significantly lower than group C ($p < 0.05$). There was a significant increase in monocyte count of the SCV group in comparison with the S group ($p < 0.05$).

The percentage of basophil in all sensitized groups was significantly higher than group C ($p < 0.05$). There was a non-significant decrease in basophil percentage of the SCV group in comparison with the S group (Table 2).

Administration of MSCs Could Repopulate the $CD4^+$ and $CD8^+$ Cells in Asthmatic Rats

Based on our results, no significant modulatory effects of either cell administration or CM injection were achieved on the population of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in non-asthmatic rats. In contrast, the percentages of $CD3^+CD4^+$ cells in all sensitized groups significantly decreased as compared with group C ($p < 0.001$) while $CD3^+CD8^+$ cells increased ($p < 0.001$ to $p < 0.05$). Of note, the levels of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in asthmatic rats that received MSCs showed a drastic difference in comparison with S and SSV groups. In detail, the percentages of $CD3^+CD4^+$ increased significantly ($p < 0.05$), whereas $CD3^+CD8^+$ cells

Table 2. Total and Differential Percentage of White Blood Cell (WBC) Count in Lung Lavage Fluid of C (Control Group), CSV (Healthy Animals Received CM), CCV (Healthy Animals Received rBMMSCs), S (Sensitized Animals), SSV (Sensitized Animals Received CM), and SCV (Sensitized Animals Received rBMMSCs) (for Each Group, $n = 6$)

	C	CSV	CCV	S	SSV	SCV
Total WBC (number/mm^3)	32.5 ± 2.14	32.5 ± 2.14	30.83 ± 2.38	100 ± 2.58 +++	97.5 ± 2.5 +++	90.83 ± 1.54 +++ * !
Eosinophil (%)	5.5 ± 0.28	5.6 ± 0.4	5.4 ± 0.44	14.88 ± 0.94 +++	13 ± 1.47 +++	10 ± 1.86 + * !
Neutrophil (%)	8 ± 0.57	8.6 ± 1.3	8 ± 0.48	14 ± 1.22 ++	15.7 ± 1.83 ++	10.67 ± 0.81 + * !
Lymphocyte (%)	68.5 ± 2.85	67.62 ± 0.89	67.86 ± 2	53 ± 2.16 ++	54.8 ± 2.44 ++	60 ± 2.24 + *
Monocyte (%)	15.32 ± 0.32	14.3 ± 0.48	15.66 ± 0.93	13.37 ± 0.65 +	14 ± 3 +	15.5 ± 4 *
Basophil (%)	2 ± 0.4	1.6 ± 0.48	2.3 ± 0.57	4 ± 0.69 +	3.2 ± 0.24 +	3.4 ± 0.36 +

Statistical differences between control and different groups: +, $p < 0.05$; ++, $p < 0.01$; +++, $p < 0.001$. Statistical differences between SSV and SCV *versus* S group: *, $p < 0.05$. Statistical differences between SSV and SCV groups: !, $p < 0.05$.

diminished simultaneously ($p < 0.01$ to $p < 0.05$). No obvious results were taken in terms of $CD3^+CD4^+$ and $CD3^+CD8^+$ cell population between SSV and S groups (Fig. 6).

Pre-Labeled Injected Cells Successfully Recruited into Pulmonary Tissue

According to immunofluorescence assay, the existence of pre-labeled MSCs was further confirmed either in non-asthmatic and peculiarly asthmatic rats that received cells systematically. Interestingly, we also affirmed the presence of injected cells in control non-asthmatic rats. It seems that MSCs could potentially recruit into normal lung tissue (Fig. 7).

DISCUSSION

In the current experiment, we evaluated the possible modulation of rBMMSC monoculture and MSC-derived CM on tracheal responsiveness and inflammatory process mainly focused on total WBC count of blood and lung lavage fluid. Additionally, the number of circulating $CD3^+CD4^+$ and $CD3^+CD8^+$ cells were further monitored.

On account of many common features of allergic asthma between rats and humans, allergic asthma induced by ovalbumin was used throughout our study [29]. Considering the characteristics of induced asthmatic sensitization, a prominent tracheal responsiveness and contractility response to methacholine with a simultaneous increase of total WBC population particularly eosinophil count followed by a marked decrement in the percentages of lymphocyte and monocyte numbers confirmed the effectiveness of sensitization in rats as compared to parallel controls [8, 30, 31].

Our results revealed that the use of rBMMSCs could improve the contractility response and tracheal responsiveness to methacholine in sensitized rats. Concurrently, the total and differential WBC counts both in the blood and lung lavage fluid nearly reached to normal state as compared to control group. With regard to different cell panels typified here, the decrement of $CD3^+CD8^+$ cells coincided with the increase in the number of $CD3^+CD4^+$ cells in cell-treated rats. Based on the given results, no obvious therapeutic effects originating from CM were also evident in ovalbumin-induced asthmatic rats. Noticeably, neither MSCs nor MSC-CM showed significant changes on the measured parameters of saline-challenged rats, which were consistent with the results of many previous studies [6]. In line with our data, the intravenous injection of BMMSCs attenuated the airway inflammation rate of an asthmatic murine model [6, 14, 32]. It seems that the extensive vascular bed, eminent production of inflammatory factors, and various chemokines through an active dynamic of inflamed sites, especially as seen in asthmatic lung, are responsible for the high rate of MSC recruitment [16, 33]. Additionally, both anti-inflammatory and immune-regulatory effects driven by MSCs have been already documented [9]. Contradicting data were, however, reported by different working groups, especially by Trzil *et al.*, that the administration of adipose-derived MSCs did

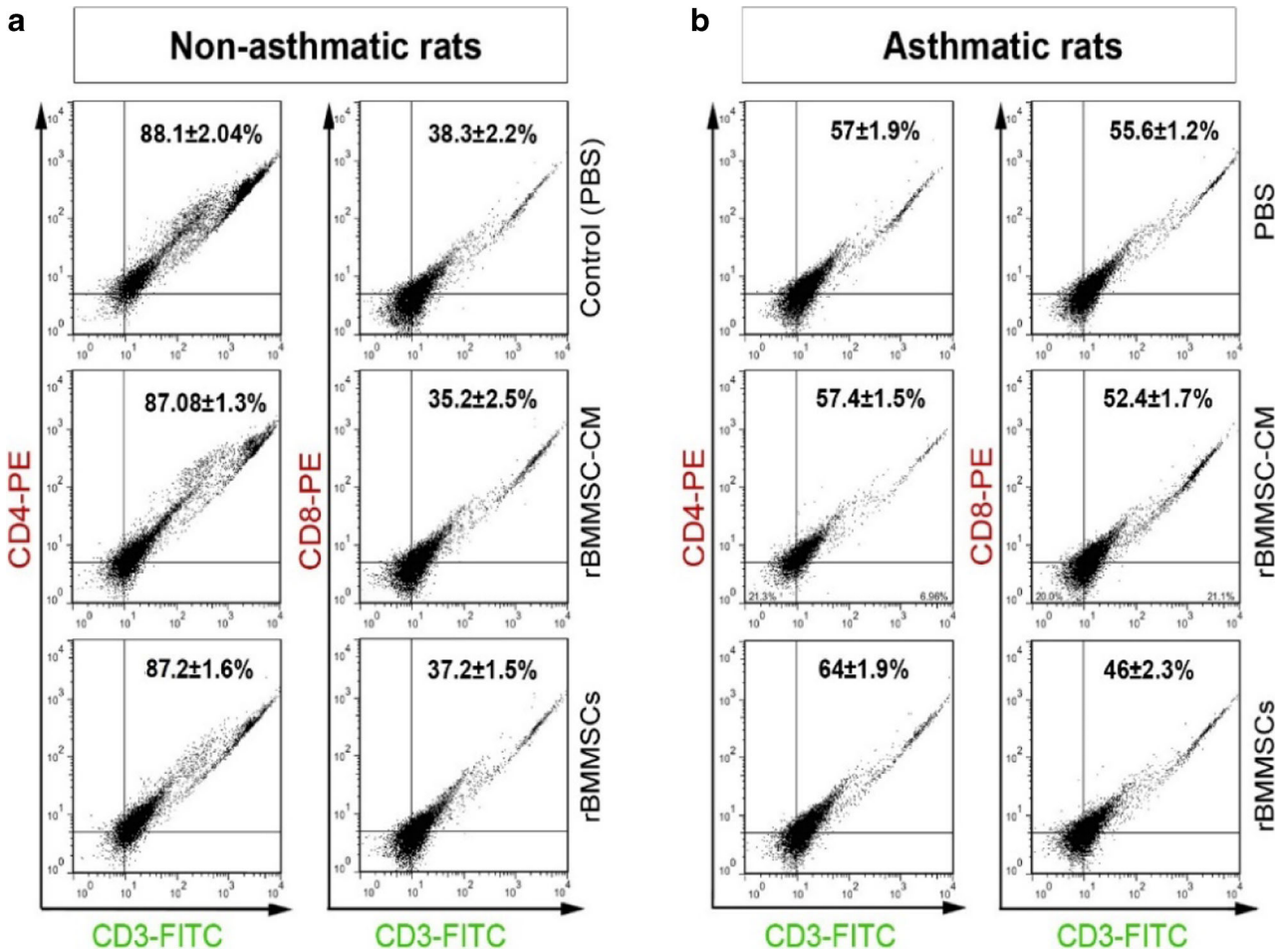

Fig. 6. Flow cytometric analysis of rat peripheral mononuclear cells in different groups (**a**, **b**). The cells were stained with a panel of antibodies, including $CD3^{-}CD4$ and $CD3^{-}CD8$. The percentage of mononuclear cells expressing each marker is expressed as mean ± SEM.

not attenuate airway inflammation on a feline model of asthma [15]. The logical explanation for this discrepancy could be presumably related to animal species, sensitization method, the source, dosage, route, and time of cell administration [34]. Also, some experiments showed that the survival and differentiation rate might be faded at sites of injury and inflammation [35–37]. In the current study, an appropriate number of pre-labeled MSCs were proved in pulmonary tissues of asthmatic rats and other groups. Some authorities also acclaimed that a distinct post-transplantation period must be assumed to track MSCs in specific tissues [38, 39]. In one study, human and mouse adipose stem cells were successfully detected 3 weeks post-transplantation [39]. Similar to work by Ghorbani *et al*., we confirmed the presence of pre-labeled cells in injured lungs after 2 weeks [38]. As expected in the distribution of vascular bed of the pulmonary system, we however detected the presence of pre-labeled cells in normal non-asthmatic rats. In contrast to our result, Abreu and other authorities revealed MSCs homing only in injured lung tissue [11, 38]. By virtue of animal strain and species, injection route, and recovery time after cell injection, stem cell type and the number of injected cells, grade and type of damaged tissue, and volume and type of cell tracker used controversial results that have been obtained [10, 15, 40–42].

According to the literature, various numbers of cells ranging from 1×10^6 to 5×10^7 had been applied [43–45] by which a systemic injection of the higher number of human or mice MSCs into a murine model not only regenerated pulmonary tissue but also increased adverse effects and mortality rate because of cell-based pulmonary

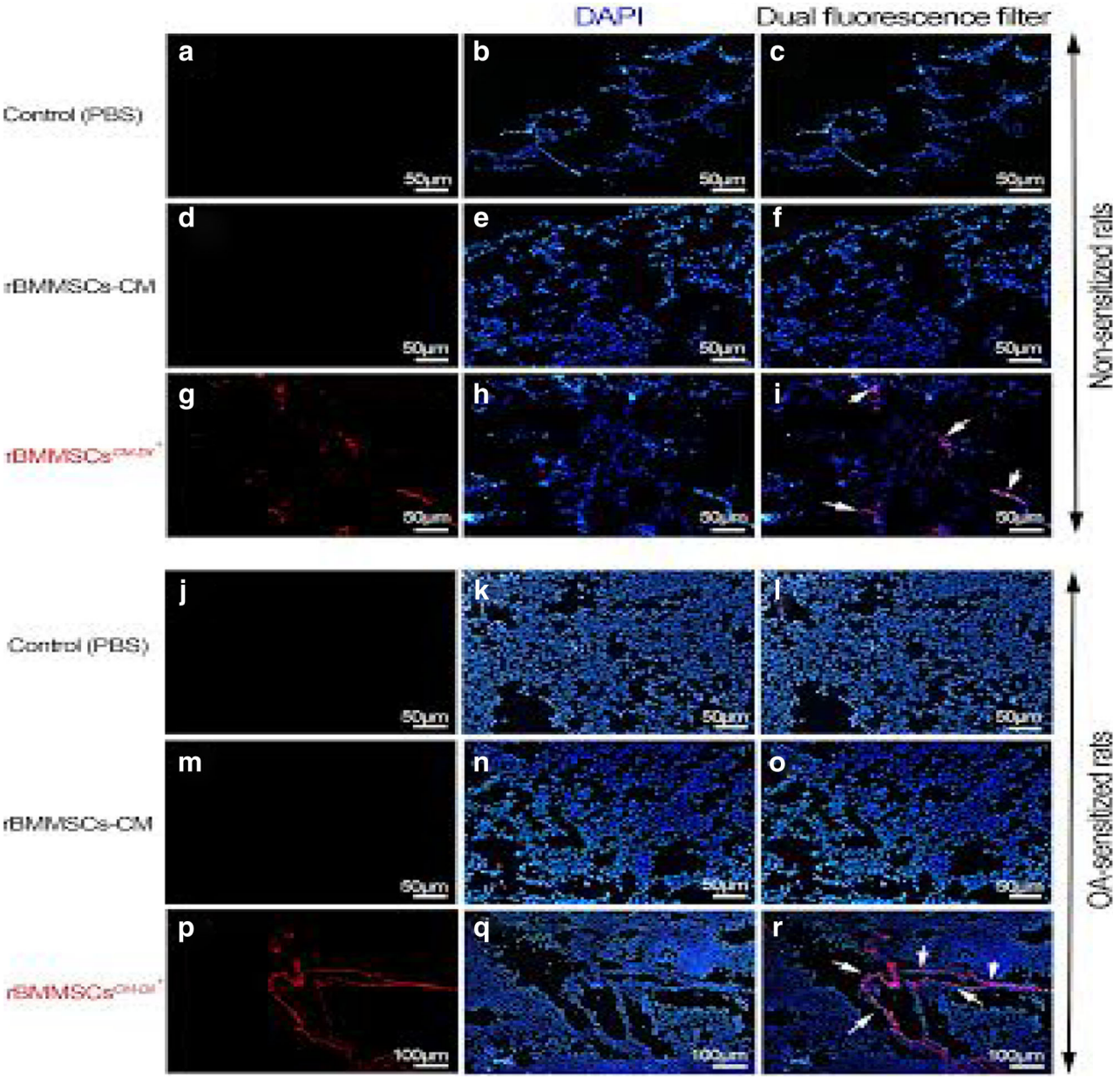

Fig. 7. Representative fluorescent image of lung parenchyma obtained from non-sensitized and sensitized rats receiving rBMMSC-CM or rBMMSCs and compared to control groups. As can be seen, the MSCs are labeled with CM-Dil and administrated intravenously.

embolism [42]. Fortunately, a well-tolerated situation without mortality or adverse effects was observed in designated experimental groups.

Our study did not unravel the paracrine therapeutic effect originated by MSCs-CM. Contrary to our result, the intravenous injection of human MSC-CM reduced apoptosis, oxidative stress, and inflammation in a porcine model of myocardial infarction and finally improved tissue function following injury [17]. Concurrently, Lonescu and his colleagues evaluated the therapeutic effect of MSC-CM on LPS-induced lung injury. Their results showed that MSC-CM decreased lung inflammation, pathological changes, and lung vascular permeability [46]. It seems that the possible reason for the ineffectiveness of systemic injection

of rBMMSCs-CM on the parameters measured for this study was a variety in secreted agents or volume used, and the condition that the CM was harvested. On the other hand, the secretome could easily disperse into the peripheral tissues and not efficiently reach injured tissue. Perhaps injection with high volume and/or repeated doses and intratracheal injection may be effective. However, further studies are suggested to evaluate the paracrine and direct effects of MSCs from other sources during different stages of induction of experimental asthma in rats.

The most beneficial effects of MSC administration seen here were devoted to MSC mobilization and recruitment into injured lungs through chemotaxis in response to inflammatory agents produced in injured tissue [47]. The prominent limitation related to our study is that the percentage of CD4 subpopulation, including T helper 1, 2 and T regulatory, was not defined. Moreover, the transdifferentiation capacity of recruited cells into pulmonary resident cells was not investigated. In conclusion, the results from this study showed anti-inflammatory therapeutic effects following intravenous administration of rBMMSCs on experimental sensitized rats. However, no regenerative responses were observed post-systemic administrations of MSC-CM.

ACKNOWLEDGMENTS

This is a report of a database from the thesis entitled "The effect of mesenchymal stem cells or their secretions on tracheal responsiveness and lung inflammation in ovalbumin-sensitized male rats" registered in Drug Applied Research Center of Tabriz University of Medical Sciences.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. None declared.

REFERENCES

1. Campos, H.D.S., and A.C.M. Lemos. 2009. Asthma and COPD according to the pulmonologist. *Jornal Brasileiro de Pneumologia* 35(4): 301–309.
2. Neamati, A., M.H. Boskabady, N. Mahdavi-Shahri, and M. Mahmoudabady. 2013. The preventive effect of *Brassica napus* L. oil on pathophysiological changes of respiratory system in experimental asthmatic rat. *Avicenna Journal of Phytomedicine* 3(1): 56.
3. Bloemen, K., S. Verstraelen, R. Van Den Heuvel, H. Witters, I. Nelissen, and G. Schoeters. 2007. The allergic cascade: review of the most important molecules in the asthmatic lung. *Immunology Letters* 113(1): 6–18.
4. Ishmael, F.T. 2011. The inflammatory response in the pathogenesis of asthma. *The Journal of the American Osteopathic Association* 111(11_suppl_7): S11–S17.
5. Douglas, G., B. Higgins, N. Barnes, A. Boyter, S. Burge, C. Cates, et al. 2008. British guideline on the management of asthma: a national clinical guideline. *Thorax* 63(Suppl. 4): iv1–iv121.
6. Bonfield, T.L., M. Koloze, D.P. Lennon, B. Zuchowski, S.E. Yang, and A.I. Caplan. 2010. Human mesenchymal stem cells suppress chronic airway inflammation in the murine ovalbumin asthma model. *American Journal of Physiology—Lung Cellular and Molecular Physiology* 299(6): L760–L770.
7. Rana Keyhanmanesh, L.P., H. Omrani, Z. Mirzamohammadi, and A.A. Shahbazfar. 2014. The effect of single dose of thymoquinone, the main constituents of *Nigella sativa*, in guinea pig model of asthma. *BioImpacts: BI* 4(2): 75.
8. Vosooghi, S., M. Mahmoudabady, A. Neamati, and H. Aghababa. 2013. Preventive effects of hydroalcoholic extract of saffron on hematological parameters of experimental asthmatic rats. *Avicenna Journal of Phytomedicine*. 3(3): 279.
9. Kavanagh, H., and B. Mahon. 2011. Allogeneic mesenchymal stem cells prevent allergic airway inflammation by inducing murine regulatory T cells. *Allergy* 66(4): 523–531.
10. Abreu, S.C., M.A. Antunes, T. Maron-Gutierrez, F.F. Cruz, D.S. Ornellas, A.L. Silva, et al. 2013. Bone marrow mononuclear cell therapy in experimental allergic asthma: intratracheal versus intravenous administration. *Respiratory Physiology & Neurobiology* 185(3): 615–624.
11. Abreu, S.C., M.A. Antunes, J.C. de Castro, M.V. de Oliveira, E. Bandeira, D.S. Ornellas, et al. 2013. Bone marrow-derived mononuclear cells vs. mesenchymal stromal cells in experimental allergic asthma. *Respiratory Physiology & Neurobiology* 187(2): 190–198.
12. da Silva Meirelles, L., P.C. Chagastelles, and N.B. Nardi. 2006. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *Journal of Cell Science* 119(11): 2204–2213.
13. Bydlowski, S.P., A.A. Debes, L.M. Maselli, and F.L. Janz. 2009. Características biológicas das células-tronco mesenquimais. *Revista Brasileira de Hematologia e Hemoterapia* 31(supl 1): 25–35.
14. Goodwin, M., V. Sueblinvong, P. Eisenhauer, N.P. Ziats, L. LeClair, M.E. Poynter, et al. 2011. Bone marrow-derived mesenchymal stromal cells inhibit Th2-mediated allergic airways inflammation in mice. *Stem Cells* 29(7): 1137–1148.
15. Trzil, J.E., I. Masseau, T.L. Webb, C.H. Chang, J.R. Dodam, L.A. Cohn, et al. 2014. Long-term evaluation of mesenchymal stem cell therapy in a feline model of chronic allergic asthma. *Clinical and Experimental Allergy*. 44(12): 1546–1557.
16. Kyurkchiev, D., I. Bochev, E. Ivanova-Todorova, M. Mourdjeva, T. Oreshkova, K. Belemezova, et al. 2014. Secretion of immunoregulatory cytokines by mesenchymal stem cells. *World Journal of Stem Cells* 6(5): 552.
17. Timmers, L., S.K. Lim, I.E. Hoefer, F. Arslan, R.C. Lai, A.A. van Oorschot, et al. 2011. Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction. *Stem Cell Research* 6(3): 206–214.
18. Rahbarghazi, R., S.M. Nassiri, P. Khazraiinia, A.-M. Kajbafzadeh, S.H. Ahmadi, E. Mohammadi, et al. 2012. Juxtacrine and paracrine interactions of rat marrow-derived mesenchymal stem cells, muscle-derived satellite cells, and neonatal cardiomyocytes with endothelial cells in angiogenesis dynamics. *Stem Cells and Development* 22(6): 855–865.

19. Rahbarghazi, R., S.M. Nassiri, S.H. Ahmadi, E. Mohammadi, S. Rabbani, A. Araghi, et al. 2014. Dynamic induction of pro-angiogenic milieu after transplantation of marrow-derived mesenchymal stem cells in experimental myocardial infarction. *International Journal of Cardiology* 173(3): 453–466.
20. Mao, Q., C.-X. Lin, X.-L. Liang, J.-S. Gao, and B. Xu. 2013. Mesenchymal stem cells overexpressing integrin-linked kinase attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions. *Molecular Medicine Reports* 7(5): 1617–1623.
21. Schuster, M., T. Tschernig, N. Krug, and R. Pabst. 2000. Lymphocytes migrate from the blood into the bronchoalveolar lavage and lung parenchyma in the asthma model of the brown Norway rat. *American Journal of Respiratory and Critical Care Medicine* 161(2): 558–566.
22. Khazdair, M.R., M.H. Boskabady, A. Tabatabaee, M. Hosseini, and M. Abbasnejad. 2013. Effect of inhaled fluticasone and salmeterol on tracheal responsiveness and lung inflammation: influence of administration time and allergen-free period. *Indian Journal of Medical Sciences* 67(3): 78.
23. Gholamnezhad Z, Boskabady MH, Khazdair MR, Hosseini M, Abbasnejad M. 2014. Effect of fluticasone and salmeterol on tracheal responsiveness to ovalbumin and lung inflammation, administrated during and after sensitization. The Scientific World Journal. 2014.
24. Hajighahramani, S., and N. Vesal. 2007. Evaluation of several drug combinations for intraperitoneal anaesthesia in adult male rats. *Iranian Journal of Veterinary Research*. 8(2): 106–115.
25. Chang, Y.-N., C.-H. Wang, C.-C. Wu, and H.-W. Wang. 2012. Effects of bupivacaine on the isolated rat tracheal smooth muscle. *Journal of Experimental and Clinical Medicine* 4(1): 62–65.
26. Keyhanmanesh, R., H. Bagban, H. Nazemiyeh, F.M. Bavil, M.R. Alipour, and M. Ahmady. 2013. The relaxant effects of different methanolic fractions of *Nigella sativa* on guinea pig tracheal chains. *Iranian Journal of Basic Medical Sciences* 16(2): 123.
27. Boskabady, M.H., R. Keyhanmanesh, S. Khamneh, and M.A. Ebrahimi. 2011. The effect of *Nigella sativa* extract on tracheal responsiveness and lung inflammation in ovalbumin-sensitized guinea pigs. *Clinics* 66(5): 879–887.
28. Mahmoudabady, M., A. Neamati, S. Vosooghi, and H. Aghababa. 2013. Hydroalcoholic extract of *Crocus sativus* effects on bronchial inflammatory cells in ovalbumin sensitized rats. *Avicenna Journal of Phytomedicine*. 3(4): 356–363.
29. Kucharewicz, I., I. Kasacka, D. Pawlak, A. Tankiewicz-Kwedlo, B. Mroczko, W. Buczko, et al. 2008. The concentration of kynurenine in rat model of asthma. *Folia Histochemica et Cytobiologica* 46(2): 199–8.
30. Pejman, L., H. Omrani, Z. Mirzamohammadi, A.A. Shahbazfar, M. Khalili, and R. Keyhanmanesh. 2014. The effect of adenosine A2A and A2B antagonists on tracheal responsiveness, serum levels of cytokines and lung inflammation in guinea pig model of asthma. *Advanced Pharmaceutical Bulletin* 4(2): 131.
31. Bayrami, G., and M. Boskabady. 2012. The potential effect of the extract of *Crocus sativus* and safranal on the total and differential white blood cells of ovalbumin-sensitized guinea pigs. *Research in Pharmaceutical Sciences* 7(4): 249.
32. Nemeth, K., A. Keane-Myers, J.M. Brown, D.D. Metcalfe, J.D. Gorham, V.G. Bundoc, et al. 2010. Bone marrow stromal cells use TGF-β to suppress allergic responses in a mouse model of ragweed-induced asthma. *Proceedings of the National Academy of Sciences* 107(12): 5652–5657.
33. Zhao, M.M., J.Z. Cui, Y. Cui, R. Li, Y.X. Tian, S.X. Song, et al. 2013. Therapeutic effect of exogenous bone marrow-derived mesenchymal stem cell transplantation on silicosis via paracrine mechanisms in rats. *Molecular Medicine Reports* 8(3): 741–746.
34. Rangwala, R., R. Leone, Y.C. Chang, L.A. Fecher, L.M. Schuchter, A. Kramer, et al. 2014. Phase I trial of hydroxychloroquine with dose-intense temozolomide in patients with advanced solid tumors and melanoma. *Autophagy* 10(8): 1369–1379.
35. Gallina, C., V. Turinetto, and C. Giachino. 2015. A new paradigm in cardiac regeneration: the mesenchymal stem cell secretome. *Stem Cells International* 2015(765846): 765846.
36. Cantinieaux, D., R. Quertainmont, S. Blacher, L. Rossi, T. Wanet, A. Noël, et al. 2013. Conditioned medium from bone marrow-derived mesenchymal stem cells improves recovery after spinal cord injury in rats: an original strategy to avoid cell transplantation. *PloS One* 8(8): e69515.
37. Linero, I., and O. Chaparro. 2014. Paracrine effect of mesenchymal stem cells derived from human adipose tissue in bone regeneration. *PloS One* 9(9): e107001.
38. Ghorbani, A., A. Feizpour, M. Hashemzahi, L. Gholami, M. Hosseini, M. Soukhtanloo, et al. 2014. The effect of adipose derived stromal cells on oxidative stress level, lung emphysema and white blood cells of guinea pigs model of chronic obstructive pulmonary disease. *Daru* 22(1): 22–26.
39. Schweitzer, K.S., B.H. Johnstone, J. Garrison, N.I. Rush, S. Cooper, D.O. Traktuev, et al. 2011. Adipose stem cell treatment in mice attenuates lung and systemic injury induced by cigarette smoking. *American Journal of Respiratory and Critical Care Medicine* 183(2): 215–225.
40. Herzog, E.L., J. Van Arnam, B. Hu, and D.S. Krause. 2006. Threshold of lung injury required for the appearance of marrow-derived lung epithelia. *Stem Cells* 24(8): 1986–1992.
41. Abe, S., C. Boyer, X. Liu, F.Q. Wen, T. Kobayashi, Q. Fang, et al. 2004. Cells derived from the circulation contribute to the repair of lung injury. *American Journal of Respiratory and Critical Care Medicine* 170(11): 1158–1163.
42. Lee, R.H., A.A. Pulin, M.J. Seo, D.J. Kota, J. Ylostalo, B.L. Larson, et al. 2009. Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. *Cell Stem Cell* 5(1): 54–63.
43. Shigemura, N., M. Okumura, S. Mizuno, Y. Imanishi, T. Nakamura, and Y. Sawa. 2006. Autologous transplantation of adipose tissue-derived stromal cells ameliorates pulmonary emphysema. *American Journal of Transplantation* 6(11): 2592–2600.
44. Ishizawa, K., H. Kubo, M. Yamada, S. Kobayashi, M. Numasaki, S. Ueda, et al. 2004. Bone marrow-derived cells contribute to lung regeneration after elastase-induced pulmonary emphysema. *FEBS Letters* 556(1): 249–252.
45. Luan, Y., X. Zhang, F. Kong, G.-H. Cheng, T.-G. Qi, and Z.-H. Zhang. 2012. Mesenchymal stem cell prevention of vascular remodeling in high flow-induced pulmonary hypertension through a paracrine mechanism. *International Immunopharmacology* 14(4): 432–437.
46. Ionescu, L., R.N. Byrne, T. van Haaften, A. Vadivel, R.S. Alphonse, G.J. Rey-Parra, et al. 2012. Stem cell conditioned medium improves acute lung injury in mice: in vivo evidence for stem cell paracrine action. *American Journal of Physiology—Lung Cellular and Molecular Physiology* 303(11): L967–L977.
47. Conese M, Piro D, Carbone A, Castellani S, Di Gioia S. 2014. Hematopoietic and mesenchymal stem cells for the treatment of chronic respiratory diseases: role of plasticity and heterogeneity. The Scientific World Journal. 2014.